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Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen, *Paenibacillus larvae*

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Abstract

Honey bee larvae are highly susceptible to the bacterial pathogen *Paenibacillus larvae* only during the first instar of larval development. Transcript levels were measured for genes encoding two antimicrobial peptides, abaecin and defensin, as well as for two candidates in the immune response cascade (PGRP-LD and *masquerade*) in control larvae and larvae exposed to the pathogen. Transcripts for all four are present throughout development. This suggests that other physiological or dietary factors may better explain the age-based change in vulnerability to this pathogen. One of these genes, abaecin, shows significant up-regulation 24 h following oral inoculation with *P. larvae*, precisely when the bacterium surmounts the midgut epithelia of bees. Expression of both antimicrobial peptides varied by 1000-fold across different nestmate bees, indicating an allelic component to their expression. The implications of these results for current hypotheses related to disease tolerance in social insects are discussed, along with implications for breeding bees resistant to this important disease.
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Keywords: *Apis mellifera*; Innate immunity; Defensin; Antimicrobial peptide; Peptidoglycan; American Foulbrood

1. Introduction

Social insects are conspicuous targets for pathogens ranging from viruses and bacteria to protozoa and fungi (Schmid-Hempel, 1998). Indeed, given their crowded living conditions and communal care, insect colonies are predicted to be especially susceptible to exploitation by pathogens, a prediction that finds strong support in honey bees (Evans and Weaver, 2003; Morse and Flottum, 1997). There are two primary routes by which social insects combat pathogens. First, colony members can minimize the spread of disease through behavioral changes including removing diseased individuals from the colony (Park, 1937; Spivak and Reuter, 2001) or changing their own behaviors when infected with pathogens (Schmid-Hempel, 1998). Second, infected colony members might resist or tolerate disease through internal defense mechanisms, including the innate-immune response. Despite substantial evidence that this response is important in reducing pathogen levels in other insects (e.g., Lowenberger et al., 1999), little is known about the role of induced immunity against honey bee pathogens. Expression levels of genes encoding disease-related proteins can help infer which proteins are involved with disease tolerance and resistance in honey bees (Evans, 2001). This, in turn, can help attempts to breed bees and other beneficial insects that are resistant to disease. It can also lay the groundwork for testing exciting theoretical advances aimed at exploring how social insect colonies survive attacks by diverse pests and pathogens (Brown and Schmid-Hempel, 2003; Schmid-Hempel, 2003; Tarpy, 2003).

Pioneering work by Tempst, Casteels, and colleagues (Casteels et al., 1989, 1990; Casteels-Josson et al., 1994) described the induction and characteristics of several antimicrobial peptides produced by honey bees. These peptides share sequence-level traits with known antimicrobial peptides from *Drosophila* and other insects (Casteels and Tempst, 1994) and have been shown to be active in vitro against both gram-positive and gram-negative bacteria as well as fungi. While functional studies have not been carried out, the antimicrobial

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peptides of bees are presumably induced through one of two immune response cascades common to insects (Tzou et al., 2002). These responses are initiated by recognition proteins which, in the presence of foreign organisms including bacteria, trigger distinct signal transduction and modulation pathways.

Unfortunately from the standpoint of understanding honey bee disease, studies to date have relied on non-native bacteria such as *Escherichia coli* to elicit immune responses. Further, these studies have used adult injections as a means of delivering immune activators, both a life stage and a mechanism that are not directly relevant for many honey bee diseases (Shimanuki, 1997). The project described here was carried out to clarify potential roles of these antimicrobial peptides during invasion by a primary natural pathogen. Bees were infected naturally (orally) by the gram-positive bacterial pathogen *Paenibacillus larvae*, the cause of the widespread larval disease American Foulbrood (Brodsgaard et al., 2000a). The importance of this pathogen to beekeepers is increasing thanks to widespread antibiotic resistance (Alippi, 1996; Evans, 2003) and the compounded impacts of other pests and pathogens (Brodsgaard et al., 2000b). Inoculated and control bees were scored for transcript levels of two key antibacterial peptides, abaecin and defensin, and two proteins predicted by sequence similarity to be early actors in the insect immune response (peptidoglycan receptor protein PGRP-LD; Werner et al., 2000, and *masquerade*; De Gregorio et al., 2001; Irving et al., 2001).

In honey bee colonies, *P. larvae* spores are transferred to young larvae during feeding by adult nestmates. These spores germinate within the larval midgut in the following 24 h, then can invade the midgut wall, enter the haemoceol, and kill larvae prior to pupation. The dried remains (scales) of dead larvae contain approximately 2×10^9 spores each, providing a potentially high source of infective material to the adult bees that remove them from the colony (Shimanuki, 1997). Adult bees complete the cycle by transmitting spores to their nestmates while exchanging food with larvae and other adults. It is well established that younger bee larvae are most susceptible to foulbrood disease. In fact, larvae infected in the third instar and beyond do not show signs of the disease (Brodsgaard et al., 1998; Shimanuki, 1997). This result could reflect (1) changes in the honey bee midgut environment that might inhibit bacterial growth (Riessberger-Galle et al., 2001), (2) a decrease in the time available for bacterial invasion and replication during bee development, or (3) an inability of younger bee larvae to mount an effective immune response against this pathogen. I explore the third hypothesis here, and present evidence that susceptible bees are in fact capable of mounting an immune response, and that these bees also show constitutive levels of immune-related transcripts.

2. Materials and methods

2.1. Insect material and rearing

To minimize genetic variation, all assays were carried out on the progeny from a single wild-mated honey bee queen (*Apis mellifera ligustica*) maintained in a disease-free apiary at the USDA Bee Research Lab, Beltsville, MD, USA. Larvae collected from this colony were reared at high humidity at 34 °C in U-shaped 96-well plates containing an excess of a liquid diet that supports larval development (Vandenberg and Shimanuki, 1988). The food was refreshed every 24 h.

Experimental larvae were inoculated with a final concentration of 100 spores/μl from pathogenic isolates of *P. larvae* mixed directly into their food (Brodsgaard et al., 1998) while controls received only the liquid diet. Four of 9 trials involved inoculation with an isolate collected in Maryland, USA (DK109), while the remaining trials involved an isolate from Berkeley, CA (BRL230010). Trials were initiated when larvae were 0–12 (early first instar), 24–36 (second instar), 48–60 (third instar), and 68–80 (fourth instar) h old, based on body size estimates. Following incubations of 3, 6, 12, 24, 48, or 96 h, inoculated and control larvae were directly frozen at −80 °C.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from individual larvae using the RNAqueous protocol (Ambion), after which RNAs were quantified by spectrophotometry. DNA was removed using a 45 min DNAse incubation at 37 °C (5 U DNAse I in appropriate buffer; Boehringer–Mannheim, with the RNAse inhibitor RNAsin; Ambion). Next, 1st-strand cDNAs by incubating approximately 2 μg total RNA at 42 °C for 1 h in a solution of 50 U Superscript II (Invitrogen), 2 nmol DNTP mix, 2 nmol poly$(dT)_{18}$, and 0.1 nmol poly$(dT)_{(12–18)}$.

2.3. Quantitative PCR amplification

DNA products were amplified in 96-well microtiter trays using specific oligonucleotide primers and an Icycler Real-Time PCR thermal cycler (Bio-Rad). Fifty microliters of reaction mixes consisted of 2 U *Taq* DNA polymerase with suggested buffer (Boehringer), 0.2 μM fluorescein, 1 mM DNTP mix, 2 mM $MgCl_2$, 0.2 μM of each primer, and a final concentration of 2.5× SYBR-Green 1. Primers and their GenBank entry numbers are shown in Table 1. Abaecin and defensin primers were designed from precursor sequences for these genes (Casteels-Josson et al., 1994). Peptidoglycan receptor proteins were identified through TBLASTN searches using their *Drosophila* counterparts (Werner et al., 2000) against an extensive EST dataset for bees (Whitfield

Table 1
Oligonucleotide primers and sequence identification for real-time quantitative RT-PCR

Primer name	Sequence (5′ to 3′)	GenBank entry
Abaecin.F	CAGCATTCGCATACGTACCA	U15954
Abaecin.R	GACCAGGAAACGTTGGAAAC	U15954
Defensin.F	TGCGCTGCTAACTGTCTCAG	U15955
Defensin.R	AATGGCACTTAACCGAAACG	U15955
AmRPS5.F	AATTATTTGGTCGCTGGAATTG	BG101562
AmRPS5.R	TAACGTCCAGCAGAATGTGGTA	BG101562
PGRP-LD.F	ACAGGTTTGATTTGGGGTGA	15357449
PGRP-LD.R	GGCAACAAGGATCAAATTGC	15357449
AmMasq.F	AAACTTTGCGCGTAGCAACT	15357748
AmMasq.R	ACTAACTCCTCGCGCTGGTA	15357748

et al., 2002). One such PGRP (PGRP-LD) was identified in this way and used here. A fourth gene, an apparent ortholog for the immune-responsive *Drosophila* gene *masquerade*, also was identified from honey bee ESTs using TBLASTN. Transcript levels for a housekeeper gene (ribosomal protein S5; Evans and Wheeler, 2000) were used to normalize against variable RNA levels. This gene is in the top 15% of genes expressed in honey bee larvae and adults, based on macro-array analyses ranking 19th in a set of 179 genes (Evans and Wheeler, 2000).

All reactions were carried out using a thermal program of 95 °C for 3 min followed by 40 cycles of (95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min 30 s). Fluorescence was measured repeatedly during the 58 °C step using appropriate laser excitation and filtration (494 and 521 nm, respectively). Melt-curve analyses were used post-run to confirm that fluorescence was the result of amplified products of the predicted size, and not from PCR artifacts such as primer dimers.

2.4. Data analyses

For each sample × primer combination, fluorescence levels were normalized within wells using average fluorescence during cycles 2–10. Threshold cycles were defined as the point when well fluorescence became greater than 10 times the mean standard deviation across all samples. Threshold cycle numbers for defensin, abaecin, PGRP-LD, and *masquerade* were then subtracted from the RPS5 threshold for each sample. This value was then scaled as a power of 1.6 (the de facto amplification × fluorescence efficiency per cycle, as estimated using cDNA of known quantities) to produce an estimate of relative cDNA abundance. Analyses of variance were carried out using starting ages, treatments, and incubation times as factors and the controlled threshold cycle as a response. Data are presented for overall mean transcript levels across all treatments. Since the threshold cycle is an exponential function of transcript level, negative standard errors are consistently smaller than positive standard errors.

3. Results

Transcript levels for the antimicrobial peptide abaecin increased significantly upon exposure of first-instar honey bees to spores of *P. larvae* (Fig. 1). Transcripts were 4.4× higher in exposed versus control larvae after 24 h, when averaged across all nine trials (ΔC_T significant by ANOVA, $n = 64$ controls, 64 treated, $p = 0.016$). In five trials for which larvae were scored after 48 and 72 h, exposed larvae showed an increase of 2.81 and 2.61 times, respectively, when compared to controls. The differences after 48 h were significant at the 0.05 level (ANOVA, $n = 50$, $p = 0.039$), while those sampled at 72 h were non-significant (ANOVA, $n = 35$, $p = 0.11$). Abaecin levels did not change significantly when older (second, third, and fourth instar) larvae were exposed to *P. larvae* (Fig. 1), regardless of incubation time. Incubation times of 3, 6, and 12 h were not sufficient to generate a change in expression between treated and control larvae (Fig. 2).

Defensin transcripts did not change significantly as a result of exposure to the pathogen for any combination of age and incubation time (Figs. 1, 2). Nevertheless, there was a trend toward higher defensin production in three of three exposure lengths for first-instar larvae. Overall, 16 of 20 independent trials using first-instar larvae showed higher defensin levels in treated versus control samples (Sign test, Pearson's probability = 0.014).

Analyses of control larvae showed significant age-based changes for the transcript levels of abaecin and defensin (Fig. 3, ANOVA, for abaecin: F ratio = 3.515, $p = 0.015$, for defensin: F ratio = 13.8, $p < 0.001$). In both cases, the oldest larvae showed increased transcript levels. For abaecin, transcripts were significantly higher in fourth- and fifth-instar larvae than in third-instar larvae. The youngest (first-instar) larvae had 1.7-fold and 4.2-fold higher transcript levels than second and third-instar larvae, respectively. For defensin, first-, second-, and third-instar larvae all had significantly significantly lower abaecin transcript levels than did fifth-instar larvae. Generally, transcript levels for

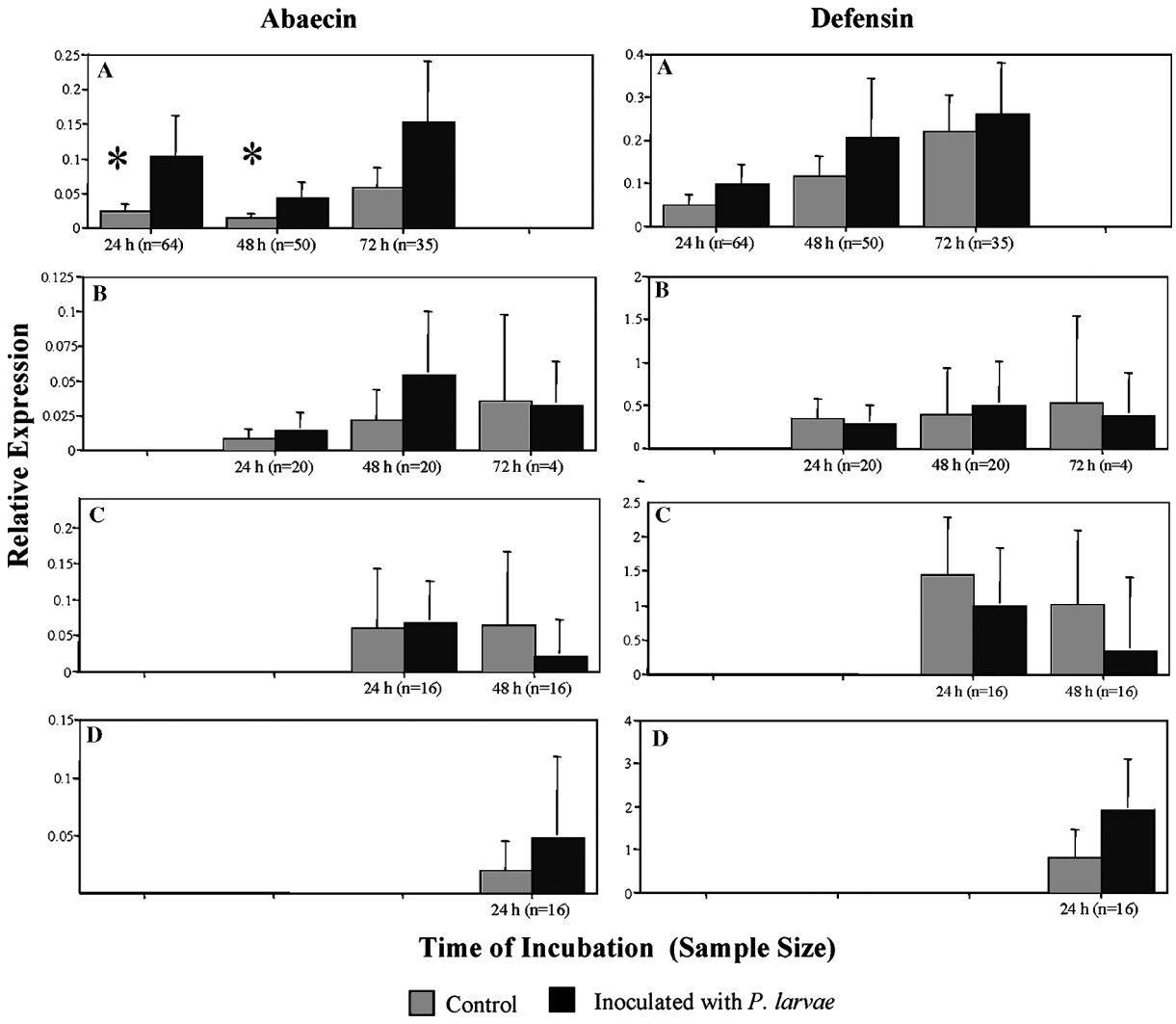

Fig. 1. Expression levels for control larvae and larvae exposed to *P. larvae* for the two antimicrobials abaecin and defensin. Larvae were inoculated as (A) first instar, (B) second instar, (C) third instar, and (D) fourth instar larvae, then were incubated for 24, 48, and 72 h. Incubation times are offset in the four trials to align (vertically) larvae of the same developmental age. Positive standard errors shown above each column. *Significant by ANOVA at $p < 0.05$.

defensin were higher than for abaecin at each age (Figs. 1 and 3, note different axes).

The results show substantial variation among individuals for immune-gene expression, even within treatments and ages. Standard deviations for the ΔC_T of first-instar controls were 6.8 for abaecin (against a mean of $= -7.98$, $n = 64$), and 5.9 (mean $= -6.31$, $n = 60$) for defensin. When normalized and transformed to actual transcript levels, this suggests that a single standard-deviation spread around the mean (~68% of samples) would range from 0.001 to 0.54 for abaecin levels and 0.003 to 0.81 for defensin. In fact individual bees of the same age and experimental treatment differed by >1000-fold in transcript levels for these two genes. Variance in abaecin and defensin transcripts was equally high in both exposed and control larvae (Brown–Forsythe test for unequal variances, for 24-h incubation $df = 124$, F ratio $= 1.63$, ns).

Bees showed a similar immune response to the two different bacterial isolates used. For first-instar larvae exposed for 24 h, abaecin levels increased by 4.8× after exposure to DK109 ($n = 16$ controls, 16 treated, SE $= 2.04$) and by 3.8× after exposure to BRL230010 ($n = 48$ each, SE $= 1.79$).

Two predicted precursors in the immune-response pathway, PGRP-LD and *masquerade*, also were expressed at all ages (Fig. 4). Neither PGRP-LD nor *masquerade* increased significantly in transcript level upon exposure to *P. larvae*, although there was a trend toward higher expression of masquerade at 24 h post-infection. Overall expression of *masquerade* decreased by 3.7-fold from the first to third instar in controls and by 40-fold over the same period in bees exposed to the bacterial pathogen, a highly significant change in the latter case (t test, $p < 0.001$).

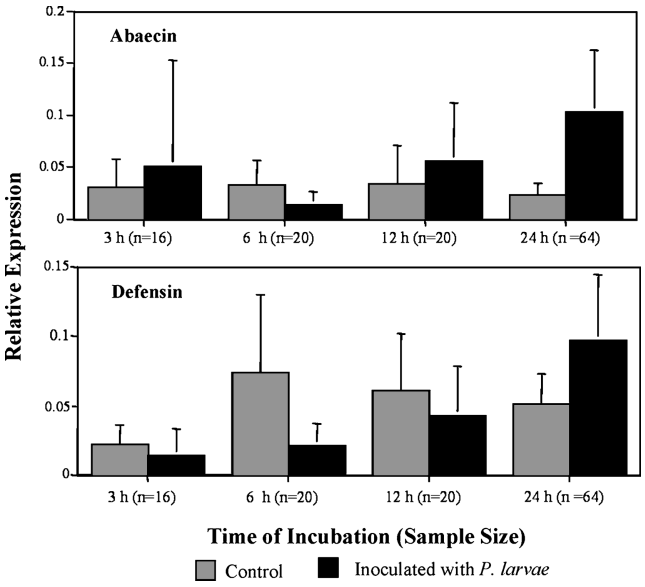

Fig. 2. Expression patterns for abaecin and defensin in first-instar larvae incubated for 3, 6, 12, and 24 h. Positive standard errors shown above each column.

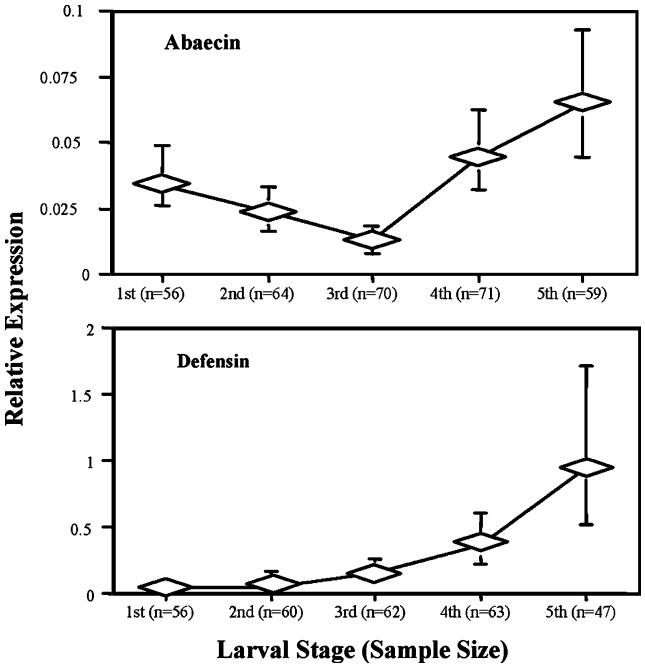

Fig. 3. Constitutive levels of abaecin and defensin in control larvae across the five larval instars. SE shown, negative SE is smaller than positive since the empirical data were exponential functions of relative expression.

4. Discussion

A primary goal of this project was to define genes in the honey bee immune response that are responsive to natural infections by the key bacterial pathogen, *P. larvae*. Among the candidates tested, the antimicrobial peptide abaecin appears to be a good marker for the progression of *P. larvae* in honey bees. The first significant up-regulation of abaecin occurred 24 h after bees were exposed to *P. larvae* spores, the point at which *P. larvae* spores have germinated and the bacterium has begun to invade the gut epithelia of larval bees (Gregorc and Bowen, 1998). Abaecin levels did not change when older larvae were exposed to the pathogen, suggesting that the disease response requires some activity on the part of the pathogen (e.g., germination or invasion of gut tissues) that is reduced in older bee larvae. These results point toward abaecin as a good indicator of successful early invasion by *P. larvae*. A second antimicrobial peptide, defensin, also appears to be responsive to *P. larvae* infection, though in a less consistent fashion.

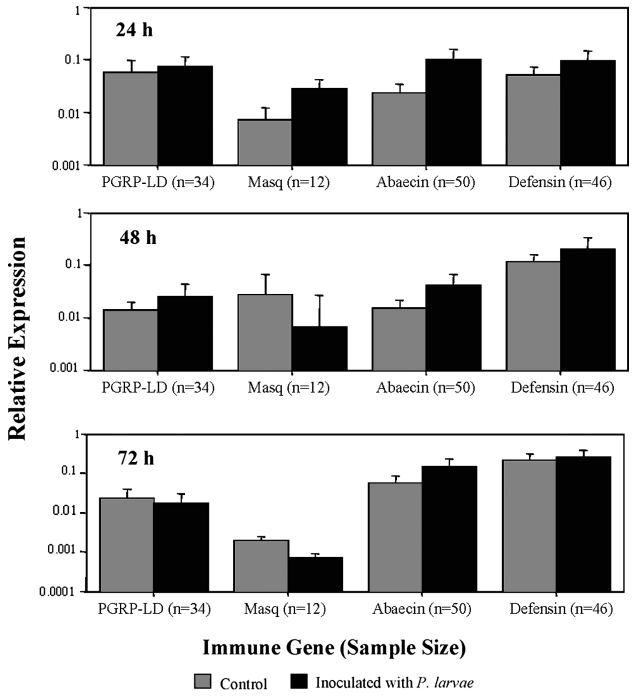

Fig. 4. Expression levels in control and exposed larvae for two immune-system candidates (PGRP-LD and *masquerade*) and two antimicrobial peptides (abaecin and defensin). First instar larvae were incubated 24, 48, and 72 h prior to analysis. Positive standard errors shown above each column.

Larval honey bees showed transcripts for both abaecin and defensin during the period at which they were most susceptible to infection by the bacterium *P. larvae*. In fact, transcription of the gene encoding abaecin, a peptide known to be effective against gram-positive bacteria (Casteels et al., 1990), was higher during the susceptible (firs-instar) stage than during the subsequent two instars, even in bees not exposed to bacteria. While defensin requires post-translational processing for activity (Casteels-Josson et al., 1994), there is no evidence that such processing is hindered in young larvae or, for that matter, age-biased in any way.

Consequently, it appears likely that antibacterial peptides in bees are available for fighting *P. larvae* early in development, the window during which this pathogen must become established in order for disease symptoms to occur. Alternate hypotheses to explain the increased susceptibility of the youngest honey bee larvae to this pathogen, including a developmental change in the environment of the larval gut (e.g., Riessberger-Galle et al., 2001), or a weakness in the gut wall of young larvae, therefore seem to be better explanations for the narrow developmental window during which this pathogen must become established.

The extent to which both control and exposed bees vary with respect to expression levels of antimicrobial peptides is striking. Control larvae, for example, showed >1000-fold differences in transcript abundance for abaecin. This variation could reflect conditional differences including the presence of undetected diseases in some larvae or differences in rearing conditions (Rinderer et al., 1974). However, the assays were restricted to members of a single bee colony which has shown no signs of bacterial, fungal or viral diseases for the past three years. Consequently, it seems more likely that much of the observed variation reflects allelic differences among colony members (either from the queen or from her multiple mates). There is both historical (Park, 1937) and recent (Palmer and Oldroyd, 2003) evidence that honey bee larvae vary in susceptibility to invasion by *P. larvae*. The results shown here suggest that this variation may arise, in part, through variation in the ability of bees to generate antimicrobial peptides. It will be especially interesting to determine whether both constitutive and induced levels of these peptides co-vary across bee genotypes, since this would help resolve whether a failure in the bee response represents a failure in pathogen recognition, signal processing, or in the actual synthesis of effectors such as abaecin that fight disease. Regardless, the results lend support to recent theoretical (Brown and Schmid-Hempel, 2003) and empirical (Tarpy, 2003) results suggesting a role for genetic diversity in mitigating disease pathologies in honey bees and other social insects. Specifically, if colony members vary in their abilities to produce antimicrobial peptides, perhaps due to high costs involved with such production or due to failure to recognize specific pathogens, the rate at which colonies succumb to disease could be reduced through the presence of diverse genotypes.

Neither of the potential immune-pathway genes assayed showed significant changes in expression after exposure to *P. larvae*. PGRP-LD transcripts are constitutively present in *Drosophila melanogaster* (Werner et al., 2000), but several members of this group show increased transcript levels upon exposure to pathogens (De Gregorio et al., 2001; Irving et al., 2001). Other members of the PGRP family (Werner et al., 2000) might prove to be better candidates as immune-responsive genes in honey bees. The results are more surprising for the inferred ortholog of *masquerade*. This gene shows 10- to 60-fold increased expression following exposure to natural or surrogate pathogens in *D. melanogaster* (De Gregorio et al., 2001; Irving et al., 2001) and is predicted to play an early role in the innate-immune response. It is possible that an immune-response role has been lost for *masquerade* in bees, or that the response of bees to *P. larvae* follows an alternate pathway.

This study underscores the potential of in vitro assays of honey bees (Brodsgaard et al., 1998) for understanding the etiology of disease. It also describes a direct way to assess the immune responses of bees to *P. larvae* and other pathogens. Knowing which components of the honey bee immune response are both necessary and subject to genetic variation should aid breeding schemes for producing bees that resist disease. More generally, the honey bee immune response offers a tractable system for assessing immune responses to a set of well-studied bacterial, fungal, viral, and eukaryotic pathogens (Evans and Weaver, 2003).

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